

**REMARKS**

With this amendment, claims 44 and 53 are pending.

Applicants have canceled claims 1-43, 45-52, and 54-62, which are drawn to non-elected inventions.

Applicants have amended claims 44 and 53. The claim amendments are fully supported by the specification. No new matter has been introduced. In particular, support for the amendment to claim 44 can be found, for example, in claim 38 as originally pending, at page 86, last two paragraphs, and at page 83, lines 5-6. Applicants have amended claim 53 to depend from pending claim 44, the only independent claim still pending.

Applicants have made these amendments solely to expedite prosecution of the present application. Applicants reserve the right to further prosecute the claims of the canceled subject matter in one or more patent applications claiming benefit to this application.

Applicants respectfully request reconsideration of amended claims 44 and 53.

**Claim Rejections Under 35 U.S.C. §112, First Paragraph – Written Description**

Claims 44 and 53 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner states that “claims 44 and 53 are directed to encompass compounds of formula II disclosed as related to valproic acid of the formula II in terms of optional groups (e.g., X, R, R1) (citation omitted), which only correspond in some undefined way to specifically instantly disclosed chemicals.” The Examiner further states that the specification does not provide structure-function data for compounds that modulate metabotropic

glutamate receptor (mGluR) activity or compounds that modulate pituitary adenylyl cyclase activator polypeptide (PACAP) expression. Applicants traverse.

This application satisfies the § 112 written description requirement and shows that applicants had possession of the claimed invention. The application, indeed, describes the claimed invention with all of its limitations using such descriptive means as words, structures, and formulas. See, e.g., MPEP 2163. In particular, the application defines R, R<sup>1</sup> and X by reciting specific, art-recognized, and well-known chemical structures. The detailed description of the chemical structures recited in claims 44 and 53, as originally pending, can also be found under Section 6.1 (“Definitions”) of the specification. Thus, a skilled artisan clearly can “envision the detailed chemical structure of the encompassed derivatives, analogs, etc” based on the disclosure of the specification. That should be and is enough to satisfy the written description requirement of the claims compounds.

Nonetheless, solely to expedite prosecution, applicant has amended pending claims 44 and 53 to recite Valproic acid and closely related compounds, i.e., the compounds of formula II wherein X is –OH, –O-alkali metal, –NH<sub>2</sub>, or –SH; and R is –CH[(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>. The Examiner has acknowledged that application’s disclosure of valproic acid “meet[s] the written description and enablement provisions of 35 USC 112” (Office Action, page 3). The other recited compounds of amended claim 44 are also supported. They are well known derivatives of Valproic acid. See also, page 86, first full paragraph of the specification. Therefore, the amended claims meet the written description requirement.

Reconsideration and withdrawal of the rejection are respectfully requested.

**Claim Rejections Under 35 U.S.C. §112, First Paragraph – Enablement**

Claims 44 and 53 stand rejected under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement. The Examiner states that “the specification, while being enabling for in vitro methods for screening compounds for utility in promoting cognitive function and preserving cognitive function in a rat, does not reasonably provide enablement for drugs, except ceftriaxone, for preserving cognitive function in certain mammals such as humans.” The Examiner also argues that “[t]o the extent that the cognitive abilities of humans is reasonably considered to be of high order as compared to rodents, one skilled in the art would not be able to predictably extrapolate the disclosed teachings of the claimed invention to humans without undue experimentation.” The Examiner further contends that the claims are relatively broad, as reading on “any subject regardless of age,” and the working examples “are limited to in vitro studies and in vivo rat studies.” Applicants traverse.

Solely to expedite prosecution, applicants have amended the claims to recite compounds of formula II wherein X is –OH, –O-alkali metal, –NH<sub>2</sub>, or –SH; and R is –CH[(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>. Applicants have also amended the claims to recite a method of attenuating the effects of age-associated cognitive impairment. The specification enables the amended claims. The application teaches that Valproic acid and related compounds can modulate expression of a gene associated with age-associated cognitive impairment (e.g., page 86). The specification further teaches well-known and accepted techniques for formulation and administration of pharmaceutical compositions comprising one or more active ingredients (e.g., page 88-91). Methods of administration and dosage information are also provided (e.g., pages 89-93). Finally, Example 9.3 provides a specific working example showing the effect of Ceftriaxone (not Valproic acid) treatment on cognitive

function in age-impaired rats. Therefore, the application provides sufficient details and working examples that enable one of ordinary skill in the art to practice the claimed methods as recited in the amended claims.

The application also provides sufficient details that enable one of ordinary skill in the art to practice the claimed methods with compounds other than Ceftriaxone. In fact, subsequent publications have confirmed the disclosure of the application, see, e.g., Exhibit B (which is a copy of a poster presented by Koh et al.), titled “Sodium Valproate Treatment Restores Cognitive Function in Aged Rat and Upregulates Endoplasmic Reticulum Chaperone, BiP, in the Hippocampus”). Figure 1 of the poster shows that sodium valproate attenuating the effects of age-associated cognitive impairment in accepted age-impaired rat models. As compared to the vehicle control (VEH), the cognitively-impaired aged group demonstrated improved memory when being treated with 100 mg/kg/day of sodium valproate (VPA 100). In particular, after a 6-hour delay, the VPA 100 group were significantly more accurate at locating the escape platform during retention trials as assessed by path length (Figure 1A) and escape latency (Figure 1B), as compared to their VEH counterparts.

Although the cognitive abilities of humans are more advanced and complex than rodents, those of ordinary skill in the art have long recognized that data obtained from in vivo rat models (such as the age-impaired rat model disclosed in the application) reasonably correlate with pharmacological effects in human. For example, a 1997 review article by Gallagher (“Animal Models of Memory Impairment,” *Memory and Aging*, 1997 vol. 352, 1711-1717, attached as Exhibit A) summarizes the value of animal models (in particular, the use of the Long-Evans rat model of spatial learning and memory to study cognitive decline in aging) as follows:

Animal models have provided a rich source of information about the neurobiological basis of memory. This presentation has focused on new insights from the use of an animal model of spatial learning and memory [i.e., the Long-Evans rat model] to study the neurobiological basis of cognitive decline in ageing. ... A clear implication from this research is that interventions designed to address cholinergic deficiency in ageing and Alzheimer's disease might be more therapeutic in treating deficits in attention than deficits in memory (see Sahakian et al. (1993) for research consistent with this proposal). Further use of well-characterized animal models will undoubtedly play a central role in determining those neurobiological effects of ageing that are important substrates for specific aspects of cognitive decline in ageing and in pathological conditions that occur in the elderly population. Such research is of clear fundamental importance in the development of appropriate and effective treatments in those conditions.

Exhibit A, Page 1716.

The application itself also describes in detail the advantage and reliability of the age-impaired Long-Evans rat model in predicting responses in humans. For example, the specification teaches that:

Features that characterize cognitive impairments in animal models likely extend to cognitive impairments in humans. ... An important feature of this model is that it mirrors the phenomenon of variability in cognitive decline among elderly humans. Furthermore, the individual differences in cognitive decline in aged rats in this model are seen in a behavioral assessment that is sensitive to the function of interconnected structures in the medial temporal lobe, a system that is essential for declarative memory in humans.

Page 2, lines 4-16 (emphasis added).

The specification further teaches that the age-impaired rat model is also useful to investigate genes that contribute to age-associated cognitive impairment in humans (page 2, last paragraph), and that “[i]n addition to reliability, the cognitive assessment used in this model has proven sensitive to effects of aging on relevant brain systems” (page 4, last paragraph).

Thus, the age-impaired Long-Evans rat model is recognized in the art to predict cognitive behaviors in human and to investigate new therapeutic treatments. As the working examples of the application provide specific data based on this well-characterized rat model (see, e.g., pages 2-3, and Example 9.1.3), one skilled in the art can predictably extrapolate the disclosed teachings of the claimed invention to humans without undue experimentation. The working examples, combined with the rest of the disclosure, enable one skilled in the art to practice the methods of the amended claims as currently pending.

For the reasons presented above and in view of amended claims 44 and 53, Applicants request that the Examiner reconsider and withdraw the § 112 rejection.

#### **Claim Rejection – 35 U.S.C. § 102(e)**

Claims 44 and 53 stand rejected under 35 U.S.C. 102(e) as allegedly being anticipated by Ohuchida et al. (“Ohuchida”). The Examiner states that Ohuchida discloses that pentanoic acid derivatives (e.g., valproic acid) are useful in improving functional activities of astrocytes and improving GABA<sub>A</sub> receptor responses, in particular, in treating or preventing neurodegenerative disease and neuronal dysfunction. Applicants traverse.

As amended, claim 44 recites “attenuating the effects of age-associated cognitive impairment in a mammal.” Ohuchida does not anticipate the method.

Ohuchida refers only generally to improving “brain functions” (column 1, line 28; column 5, lines 37 and 43) or to improving “cerebral function” (column 27, lines 66-67). Specifically, Ohuchida refers to improving the functional activities of astrocytes and improving GABA<sub>A</sub> receptor responses (column 2, lines 9-19; column 3, lines 55-60). Finally, Ohuchida suggests that these

“improvements” “are expected to be useful” in neurodegenerative disease and neuronal dysfunction (column 31, lines 22-23).

Ohuchida never refers to improving cognitive function. It never refer to attenuating the effects of age-associated cognitive impairment. Ohuchida explains that neurons and glia are “two major structural units that form the brain.” While neurons process and transmit information, glia (including astrocytes) complement the function of neurons by supplying nutrients, eliminating wastes, etc. (column 1, lines 53-67). Thus, improving functional activities of astrocytes and improving GABA<sub>A</sub> receptor responses “are expected to be useful for neurodegenerative diseases.” As such, Ohuchida does not teach attenuating the effects of age-associated cognitive impairment on “cognitive” function.

If Ohuchida thought its astrocytes or receptor responses were useful in applicant’s claimed method, it would have said so. For that reason alone, Ohuchida does not anticipate the claimed method. Indeed, in Ohuchida, in vivo tests on a ischemia-stroke rat model (see column 30, Example 4) only measured mobility and reports that “**mobility** impairment of the learned conditional avoidance response ... was improved by the invented compound(s)” (column 31, lines 3-6). In addition, Ohuchida studied astrocyte function and GABA<sub>A</sub> receptor response using young rats (“cerebrum of neonatal rats (age: day 1),” see column 28, lines 14-17, and 64-65), not rats that had age-associated cognitive impairment. Thus, Ohuchida used a test that would not have measured the effect claimed in this application. In fact, other studies confirm that Valproic acid and its salts have an adverse effect on cognitive function, learning and memory, in your rats. See, e.g., Wu and Wang, Brain and Development, 24:82-87 (2002) (Abstract is attached herein as Exhibit C). In Wu

and Wang's work, sodium valproate was shown to "adversely affect learning and memory" in immature (21 day old) rats.

For the reasons presented above, Ohuchida does not anticipate the claims 44 and 53, as amended.

### Conclusion

The Examiner is invited to telephone the undersigned to discuss any issue pertaining to this response. Applicants request favorable consideration of the application and early allowance of the pending claims. If additional fees are due, please charge our **Deposit Account No. 18-1945**, under Order No. JHUC-008-101 from which the undersigned is authorized to draw.

Dated: November 2, 2007

Respectfully submitted,

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# Animal models of memory impairment

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## SUMMARY

Memory impairment in the elderly resembles a mild temporal lobe dysfunction. Alterations in the hippocampal formation are also a probable basis for cognitive deficits in some animal models of ageing. For example, aged rats are impaired in hippocampal-dependent tests of spatial memory. Recent studies have revealed considerable structural integrity in the aged hippocampus, even in aged rats with the most impaired spatial memory. In contrast, atrophy/loss of cholinergic neurons in the basal forebrain and deficiency in cholinergic transduction in hippocampus correlate with the severity of spatial memory impairment in aged rats. This evidence supports the longstanding view that age-related loss of memory has a cholinergic basis. In this context, it is somewhat surprising that the use of a selective cholinergic immunotoxin in young rats to further test this hypothesis has revealed normal spatial memory after removing septo-hippocampal cholinergic neurons. Young rats with immunotoxic lesions, however, have other behavioural impairments in tests of attentional processing. These lines of research have implications for understanding the neurobiological basis of memory deficits in ageing and for selecting an optimal behavioural setting in which to examine therapies aimed at restoring neurobiological function.

## 1. INTRODUCTION

Memory impairment can occur as a relatively isolated sequel to brain damage. As reviewed elsewhere in this volume, studies of human amnesia and investigations of animal models for those conditions have disclosed a wealth of information about the organization of memory in the brain (see Squire & Zola, this volume). Neurobiological systems in mammals are specialized for distinct forms of memory. In particular, components of the brain's medial temporal lobe support the formation of memories that are explicitly accessible as remembered facts and events, often referred to as declarative memory.

The study of memory, based on relatively circumscribed cases of human amnesia and related animal models, has also informed our understanding of the relevant pathology underlying the failure of memory in other conditions. A recognition that neurodegeneration occurs at several critical sites in the medial temporal lobe, including the hippocampus, in Alzheimer's disease provides a basis for loss of cognitive functions that depend on those structures (Hyman *et al.* 1984, 1987; West *et al.* 1994). Because Alzheimer's disease and other forms of dementia in the elderly occur against a background of brain ageing, recent research using animal models has also helped to distinguish between such pathological conditions and normal ageing. Recent findings, which will be highlighted in this short account of that research, provide new evidence for greater neurobiological preservation in the aged brain than was previously appreciated. Animal models also promise to advance our understanding

of the functional implications of those changes that occur in the brain during normal ageing.

## 2. ANIMAL MODEL OF HIPPOCAMPAL-DEPENDENT COGNITIVE IMPAIRMENT

Considerable advances have occurred in the development of animal models geared to examining the condition of specific neurobiological systems during ageing. An important feature of such models is that functional assessments are made on behavioural tasks that require the integrity of the neurobiological system of interest. One widely used model capitalizes on the observation that tasks measuring spatial learning and memory are particularly sensitive to both hippocampal damage (Morris *et al.* 1982) and ageing in the rat (for reviews, see Gage *et al.* 1984; Gallagher & Rapp 1997). In a frequently used water maze apparatus, young and aged rats are tested in two standardized training protocols: one that relies on the functional integrity of the hippocampal system, and another that involves many of the same sensorimotor demands but does not require the medial temporal lobe system.

The water maze apparatus consists of a large, circular pool filled with water that has been made opaque through the addition of powdered milk or some other substance. In the hippocampal-dependent 'hidden platform' version of the task, rats are trained to find a camouflaged escape platform that is positioned just below the water surface. The location of this platform remains constant from trial to trial. Because there are no local cues that mark the position

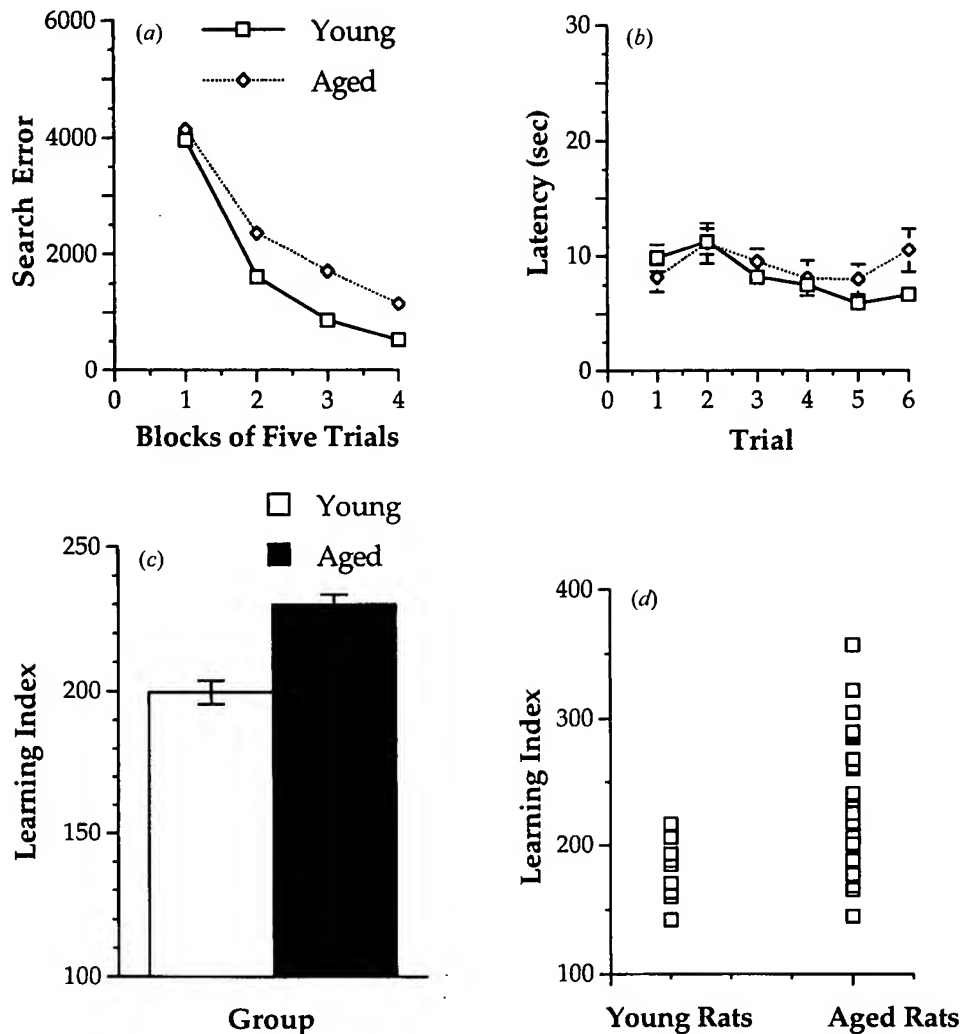


Figure 1. Water maze performance in young (6 months) and aged (28–29 months) male Long-Evans rats. (a) Age-dependent impairment during training trials in the hidden platform task that depends on spatial learning. The dependent measure, search error, is based on a calculation of average distance from the platform every second during the training trials. In contrast, aged and young rats perform with equal proficiency during cue training using a visible escape platform (shown in (b)). These graphs present archival data for 96 young and 128 aged male Long-Evans rats trained in a standard protocol described in Gallagher *et al.* (1993). Data from interpolated probe trials also show an age-dependent deficit in acquisition of a spatially guided search strategy in the hidden platform task (shown in (c)), where lower values reflect a more accurate search in the escape platform location. Individual scores derived from these probe trials are shown in the lower graph on the right (d) for a sample of young and aged rats. Note the individual differences among the aged rats, with some subjects performing as well as young and others falling outside the entire range of young performance. (With permission from Gallagher *et al.* 1993.)

of the platform, the rat's ability to locate it efficiently depends on using a spatial configuration of extramaze cues surrounding the pool. Indeed, young rats can learn to swim directly to the escape platform within relatively few training trials from any of a number of start locations at the perimeter of the pool. Probe trials are interpolated during the course of training to assess the development of a spatially guided learning strategy. At the completion of this testing rats can be assessed for their ability to locate a visible platform, unaided by spatial location within the maze environment. This cue-training version of the task is unaffected by hippocampal damage (Morris *et al.* 1982). The consistent finding across our studies, as well

as experiments conducted in other laboratories, is that a substantial proportion of aged rats exhibit behavioural deficits qualitatively similar to the effects of hippocampal damage. Other rats of the same chronological age perform as well as young rats on the hippocampal-dependent version of the water maze task. The data in figure 1 show an age-dependent impairment in spatial learning (but not cue learning) and also illustrate the phenomenon of individual differences among aged rats (Gallagher *et al.* 1993).

Behavioural assessment provides a useful context for defining neurobiological alterations in the aged brain that contribute to cognitive decline (for reviews, see Gallagher & Rapp 1997; Rapp & Amaral 1992). By

this approach, relevant neural systems in brains from aged rats with identified impairment can be compared with the condition of those systems in rats of the same age that exhibit preserved function. Such comparisons are likely to be more sensitive than merely comparing aged rats with young subjects, given that only a proportion of aged animals are impaired. In addition, it is possible that certain biological changes that occur during ageing have relatively little consequence for the maintenance of functional capacity. Thus, identification of neurobiological alterations that are closely coupled to cognitive decline may point to those effects of ageing that are likely to compromise adaptive capacities in the elderly.

### 3. PRESERVATION OF PRINCIPAL NEURONS IN HIPPOCAMPUS OF AGED RATS

The basis for age-related decline in hippocampal-dependent functions, as illustrated by spatial learning impairment, has been attributed to neurodegeneration within this system (Landfield *et al.* 1981; Meaney *et al.* 1988). Loss of neurons in the hippocampus of aged rats was originally inferred from studies that sampled neuron density in the principal cell fields of this structure. Previous studies linking neuron loss to functional decline in ageing have reported a similar result in rats characterized for spatial learning ability. For example, CA3/2 neuron density was reported to decline by as much as 30% in behaviourally impaired aged rats (Issa *et al.* 1990). The water maze task used to characterize behavioural impairment in that study was the same used to test aged rats, as shown in figure 1. Subsequent research, however, has provided compelling evidence that frank neuron degeneration is not an inevitable feature of hippocampal ageing, even in rats that exhibit substantial impairment in spatial learning ability (Rapp & Gallagher 1996; Rasmussen *et al.* 1996). In this recent work, a stereological examination yielded counts for total number of principal neurons in the hippocampus using the optical fractionator technique. For the granule cell layer, and in the CA3/2 and CA1 pyramidal cell fields, total neuron number was statistically indistinguishable in young subjects, aged rats with behavioural deficits predictive of hippocampal dysfunction and aged rats that performed as accurately as younger individuals (Rapp & Gallagher 1996). These findings indicate that previous reports of age-related changes in cell density are not likely to result from neuron loss. Although some undefined difference may exist in the aged rats used in these studies, clearly spatial learning impairment can occur in the absence of neuron loss.

The stereological results obtained in the rodent model of ageing are consistent with preliminary evidence in monkeys and humans, indicating that the principal neurons of the hippocampus are preserved in normal ageing (West 1993; West *et al.* 1993). Other recent investigations of the rodent model in our study population have indicated that a number of proteins used for neurocommunication in this system are also present in similar quantity in aged rats, irrespective of

their spatial learning ability. We have reported that the amount of hippocampal m1 and m3 receptor proteins, which serve as postsynaptic cholinergic receptors of the muscarinic type, are unaffected by age and uncorrelated with behavioural decline in ageing (Chouinard *et al.* 1995). Similarly, the amount of NR1, NR2a and NR2b protein in the hippocampus of behaviourally characterized animals is unaffected by age or behavioural status (B. B. Wolfe, personal communication). These proteins comprise subunits for a type of ionotropic glutamate receptor, known as the N-methyl-D-aspartate (NMDA) receptor, which is important for certain forms of neural plasticity in hippocampus. Indeed, the integrity of these components of hippocampal neuronal machinery is consistent with the stereological evidence that neuron numbers are preserved in this system during normal ageing.

The findings in this line of research indicate substantial structural integrity in a system where, until recently, cell loss was considered to be a major contributing factor to functional decline during the normal ageing process. The absence of neuron loss in this structure in aged subjects, contrasts with an attrition confirmed in brains from patients with Alzheimer's disease (West *et al.* 1994). Nonetheless, the behavioural decline in hippocampal-dependent learning demonstrated in the animal model would seem to indicate that other effects of ageing compromise the function of the medial temporal lobe system. Indeed, a number of alterations that could provide a basis for the emergence of behavioural deficits are evident within this circuitry. For example, stereological analyses have revealed a loss of synaptic contacts at certain sites within the hippocampal formation of aged rats (Geinisman *et al.* 1992). Impaired long-term plasticity is also evident in the aged rat hippocampus (Barnes 1979). In addition, although total numbers of principal neurons within the hippocampal formation are preserved, neurodegeneration of subcortical neurons, i.e. basal forebrain cholinergic cells, that provide input to the hippocampus has also been confirmed in aged rats (Fischer *et al.* 1991). Such effects of ageing may provide a basis for diminished proficiency in functions dependent on the hippocampal system. Indeed, a number of alterations in the hippocampal system during ageing have been found to correlate with the severity of impairment in hippocampal-dependent learning and memory, such as that observed in the spatial learning model (for review Gallagher *et al.* 1995).

### 4. RELATIONSHIP OF CHOLINERGIC NEURON LOSS TO COGNITIVE IMPAIRMENT

To establish the functional significance of a specific alteration observed in the brain during ageing, two general approaches are taken. By one approach, experimental induction of the alteration observed in ageing would be expected to produce deficits like those encountered in aged subjects. As a complementary approach, therapeutic treatments designed to remedy or compensate for a neurobiological alteration would be expected to improve deficits caused by that

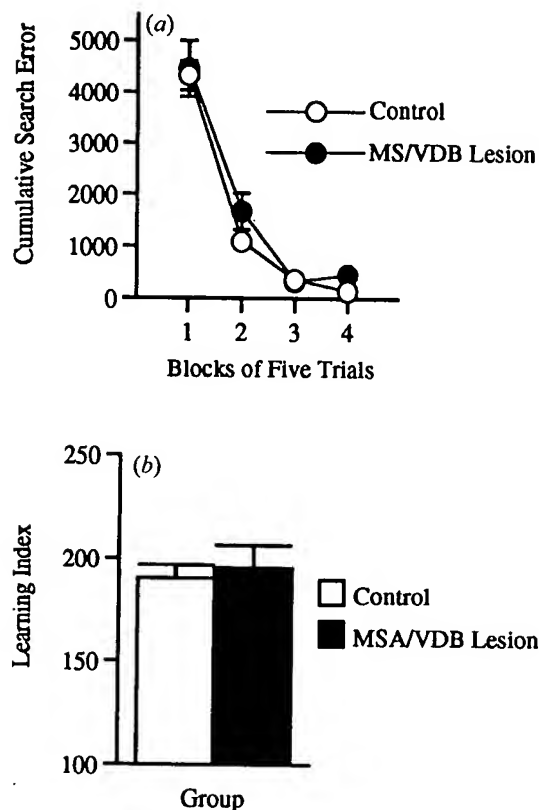


Figure 2. Effects of immunotoxic lesions of the cholinergic neurons that project to hippocampus on spatial learning in the water maze (Baxter *et al.* 1995). Both control and lesioned rats show comparable learning during training trials (a) and probe trials (b).

alteration. We now turn to some unexpected findings from studies designed to examine the contribution of cholinergic deficiency to the decline in hippocampal-dependent spatial learning observed in aged rats.

A deficiency in the cholinergic system has been of long-standing interest as a basis for memory impairment in ageing and age-related pathological conditions, such as Alzheimer's disease. The basal forebrain cholinergic system consists of a grouping of large neurons that extends from the medial septum through the diagonal band of Broca to the nucleus basalis of Meynert, also including the subnucleus substantia innominata, particularly in rodent brain (Wainer & Mesulam 1990). Neurons situated more rostrally, in the medial septum and vertical limb of the diagonal band (MS/vDB), predominantly innervate the hippocampal formation. At more caudal levels, cholinergic neurons innervate neocortex and the amygdala complex. These cholinergic neurons undergo substantial degeneration in Alzheimer's disease (Coyle *et al.* 1983; Davies & Maloney 1976) and to a lesser extent in normal ageing (Bartus *et al.* 1985). In the rodent model of hippocampal-dependent function, a number of laboratories have reported that deterioration of these cholinergic neurons is reliably correlated with age-related decline in spatial learning in rats (for reviews, see Fisher *et al.* 1991; Gallagher *et al.* 1995). Such findings are consistent with correlations also reported between the degree of

cholinergic loss and severity of cognitive decline in dementia (Bartus *et al.* 1985). All of these observations have been viewed as lending support to the concept that memory impairment in ageing and dementia is attributable to a cholinergic deficiency. By this view, it would be expected that removal of cholinergic neurons would be sufficient to cause deficits that resemble those encountered in ageing and, to a more severe degree, in dementia. A test of this prediction has become possible by the recent development of a selective toxin for cholinergic neurons.

The immunotoxin, 192 IgG-saporin, is composed of a monoclonal antibody to the 'low-affinity' p75 NGF receptor ( $I_1$ NGFr) coupled to a ribosome-inactivating cytotoxin, saporin (Wiley *et al.* 1991). In the basal forebrain of rats, the  $I_1$ NGFr is selectively expressed on cholinergic neurons, so direct infusion of 192 IgG-saporin into basal forebrain nuclei produces selective lesioning of cholinergic neurons, sparing non-cholinergic neurons at the lesion site. The lesion methods using microinjection of 192 IgG-saporin into the MS/vDB in the studies described here produced >90% reduction of ChAT activity in the hippocampal formation. If deficits in spatial learning in aged animals are the result (entirely or partially) of loss of cholinergic input to the hippocampus, young animals with this immunotoxic lesion should have impairments in spatial learning that are similar to those seen in aged rats. This hypothesis was examined by testing young immunolesioned rats in the same protocol that has been well-characterized in our laboratory for examining the deficits in spatial learning that occur in ageing (Baxter *et al.* 1995). The results from that experiment are shown in figure 2.

Surprisingly, rats with removal of the septo-hippocampal cholinergic projection acquire the spatial learning task as readily as intact rats. The failure to find substantial impairment in the spatial learning water maze task, as well as in other assessments of learning and memory, has also been reported by other investigators using 192 IgG-saporin to produce selective cholinergic lesions of components of the basal forebrain system that innervate hippocampus (Berger-Sweeney *et al.* 1994; Torres *et al.* 1994).

A selective loss of cholinergic neurons that innervate the hippocampal formation might not provide an adequate model for the effects of ageing on the basal forebrain cholinergic system. If the entire basal forebrain cholinergic system, with its collective projections to hippocampus, amygdala and cortex normally provides a coordinated regulation of certain processing capacities in limbic/cortical circuitry, then more complete removal of the entire basal forebrain cholinergic system, rather than components of it, may provide a better basis for revealing its function. More extensive lesions of the basal forebrain cholinergic system may also better mimic the pattern of loss that occurs during ageing and age-related pathological conditions. Deficiencies in the components of this system that innervate cortex are typically seen along with loss of the septo-hippocampal cholinergic projection. In a more recent study, microinjections were made throughout the rostral/caudal extent of the

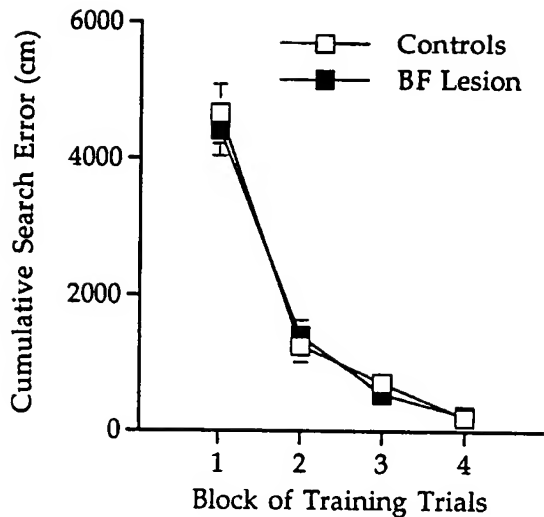


Figure 3. Effects of combined lesions of the MS/VDB and nBM/Sl (basal forebrain (BF) lesion) on spatial learning in the water maze (Baxter *et al.* 1996). Both control and lesioned rats show comparable learning.

basal forebrain system. Despite this extensive removal of the basal forebrain cholinergic system, the data in figure 3 show that spatial learning was acquired normally by the immunolesioned rats (Baxter *et al.* 1996).

Before concluding that loss of the integrity of cholinergic input to hippocampus is not sufficient to cause spatial learning impairment, we thought it important to examine the effects of removing septo-hippocampal cholinergic neurons in aged rats. Against the background of other neurobiological changes in the hippocampus of aged rats, removal of these neurons might produce a deficit where none is seen in young animals. For this purpose, young and aged rats were initially screened in our standard spatial learning protocol to select matched groups for the control and lesion conditions. Aged rats with relatively preserved learning ability were chosen so as to avoid a floor effect in our assessment of the lesion, but we also included some aged rats with marginal spatial learning impairment to determine if these rats might be more vulnerable to the effects of cholinergic lesions. Nonetheless, we found no difference in this study between lesion and control groups, irrespective of age or preoperative performance (see Baxter & Gallagher 1996, figure 4).

The experiments described thus far indicate that removal of basal forebrain cholinergic neurons is not sufficient to produce spatial learning impairment. It might be argued, however, that the contribution of these neurons to behaviour might only be detected if additional effects that coincide with greater cognitive impairment in ageing were also reproduced. Thus, the behavioural capacity of aged rats with relatively intact or only mild impairment would not be further compromised by a selective cholinergic lesion. Other studies, however, argue against this view that a cholinergic lesion, by itself, is not sufficient to cause behavioural impairment. Indeed, young rats with selective lesions

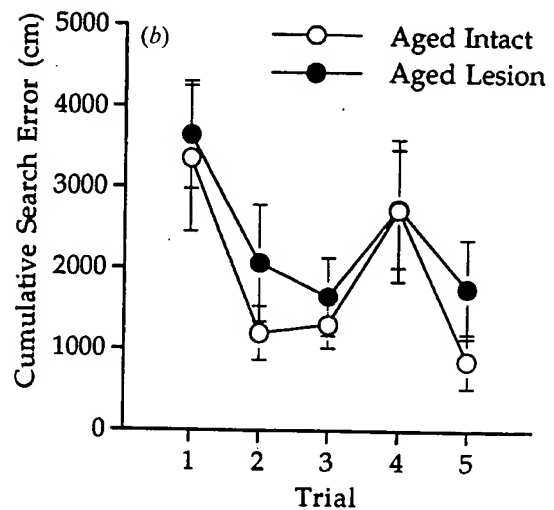
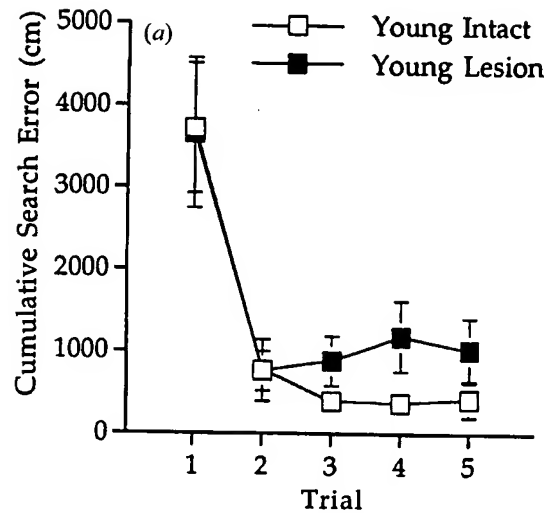


Figure 4. Effects of immunolesions removing cholinergic input to the hippocampus on spatial learning in young (a) and aged rats (b). All rats were tested in a standard water maze protocol (Gallagher *et al.* 1993) prior to assignment to lesion or control groups; rats within an age group were matched for preoperative spatial learning ability and aged rats with severely impaired spatial learning were excluded. Following surgery, spatial learning was tested in a new maze with different extra-maze cues. There was no significant effect of the immunolesion on spatial learning in either young or aged rats (Baxter & Gallagher 1996).

of the basal forebrain cholinergic system are impaired on assessments in other behavioural domains. In particular, selective removal of these cholinergic neurons in young rats using the toxin 192 IgG-saporin is sufficient to cause substantial deficits in tasks that primarily assess the regulation of attentional processes (Baxter *et al.* 1997; Chiba *et al.* 1995, 1997; and see Baxter & Gallagher 1997, for a review). Moreover, those findings indicate that lesions of different components of this system (innervating hippocampus and cortex) impair attentional processes in distinctive ways. These new findings add support to a shifting focus on the contribution of

cholinergic deficiency in ageing and Alzheimer's disease to impairments in attentional processes (Jones *et al.* 1995; Moore *et al.* 1992; Mouloua & Parasuraman 1995; Parasuraman *et al.* 1992; Parasuraman & Haxby 1993).

If removal of cholinergic neurons in the MS/vDB is ineffective for producing spatial learning deficits in young and aged rats, how are correlations between cholinergic degeneration in the MS/vDB and age-related spatial learning impairments to be interpreted? Such correlations have suggested that cholinergic deficiency contributes to the functional loss assessed in the animal model of spatial learning. An alternative view of such correlations is that age-related changes in cholinergic neurons reflect a separate, underlying condition in the hippocampus that itself causes spatial learning impairment. Indeed, selective neurotoxic lesions of hippocampus (unlike removal of the septohippocampal cholinergic neurons) do reproduce age-related deficits in spatial learning (Gallagher & Holland 1992). Consistent with this possibility, there is also extensive evidence for age-related changes in the hippocampus other than those alterations found in the cholinergic projection system (Barnes 1979; Geinesman *et al.* 1992; and Gallagher & Rapp 1997, for a review). By this view, the condition of the aged hippocampus that underlies spatial learning impairment (or some subset of those changes) would be associated only secondarily with deterioration in the cholinergic system.

## 5. CONCLUSION

Animal models have provided a rich source of information about the neurobiological basis of memory. This presentation has focused on new insights from the use of an animal model of spatial learning and memory to study the neurobiological basis of cognitive decline in ageing. Recent research has shown that identified impairment in learning and memory in aged rats is not tied to frank neurodegeneration in the hippocampal system. As an extension of the study of spatial learning in aged rodents, research using this model to assess experimental treatments that reproduce specific features of neurobiological ageing in young animals, such as deterioration in the basal forebrain cholinergic projection to hippocampus, have led to a reconceptualization of the functional significance of age-related deterioration in this system. A clear implication from this research is that interventions designed to address cholinergic deficiency in ageing and Alzheimer's disease might be more therapeutic in treating deficits in attention than deficits in memory (see Sahakian *et al.* (1993) for research consistent with this proposal). Further use of well-characterized animal models will undoubtedly play a central role in determining those neurobiological effects of ageing that are important substrates for specific aspects of cognitive decline in ageing and in pathological conditions that occur in the elderly population. Such research is of clear fundamental importance in the development of appropriate and effective treatments in those conditions.

Supported by grants from the National Institute of Mental Health (KO5-MH01149) and National Institute on Ageing (PO1-AG09973).

*Phil. Trans. R. Soc. Lond. B* (1997)

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# SODIUM VALPROATE TREATMENT RESTORES COGNITIVE FUNCTIONING IN AGED RATS AND UPREGULATES ENDOPLASMIC RETICULUM CHAPERONE, BiP, IN THE HIPPOCAMPUS

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95.7

## INTRODUCTION

- A condition of medial temporal lobe hyperfunction has been reported in fMRI studies of humans with early stages of mild cognitive impairment (eMCI; Dickerson et al., 2005; Neurology, 65:404).
- Similarly, neurons in the CA3 field of the hippocampus are hyperactive and fail to encode new information in aged rats with cognitive impairment (Wilson et al., 2005, J Neurosci, 25:6877).
- We have found that treatments aimed at reducing this hyperfunction in aged rats improved cognitive performance.
- One such treatment is via sodium valproate (VPA), an effective antiepileptic medication for limbic seizures that modifies excitatory/inhibitory functions by increasing glutamate reuptake and GABA concentrations.
- Here, we examined whether VPA treatment would increase expression of endoplasmic reticulum chaperones which are required for protein folding and protect against deleterious consequences of excitotoxicity and oxidative stress.

## MATERIALS AND METHODS

### Behavioral Experiment

Aged Long-Evans male rats (24-mo-old) that demonstrated impaired memory performance in a standardized assessment of spatial cognition using a water maze (Gallagher, Burwell, & Burchinal, 1993) were assigned to either drug or vehicle treatment. These rats were treated subcutaneously via osmotic mini-pump with VPA (50 or 100 mg/kg/day) or saline control starting 2 weeks prior to training in a new water maze environment.

We tested the effects of these drugs on the performance of aged rats in a modified water maze task. Unlike the traditional water maze protocol wherein the escape platform location remained constant throughout training, the escape platform location in this spatial memory version of the task varied from day to day. During training, rats were given 6 trials per day to locate the submerged escape platform (60 s per trial). The rats were allowed to remain on the platform for 20 s and were then placed in a holding cage for another 40 s before the next trial. At the end of the training session, the rats were returned to their home cages. After a 6 hr delay, the rats were given a retention trial (60 s) with the submerged platform located in the same position as in the training trials. Rats were trained and tested in this manner for 5 consecutive days. Performances on the last 3 days were averaged for analysis.

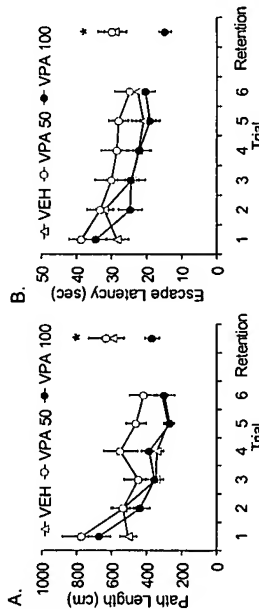
### In Situ Hybridization Experiments

Cognitively impaired aged rats (see above) were treated with VPA (100 mg/kg/day) or saline in a similar manner as described above before their brains were harvested following perfusion. BiP/GRP78 and calreticulin riboprobe templates were generated by PCR from total hippocampal RNA and modified to contain SP6 and T7 RNA polymerase sites. <sup>35</sup>S-labeled riboprobe was generated using the Maxiscript kit (Ambion). Brain sections (30 µm) containing the dorsal hippocampus were hybridized overnight at 60°C in buffer containing the radiolabeled riboprobe. Hybridized sections were then extensively washed, mounted onto slides, and quantified by PhosphorImager system (Molecular Dynamics/Amersham).

### Protein Extraction and Western Blot

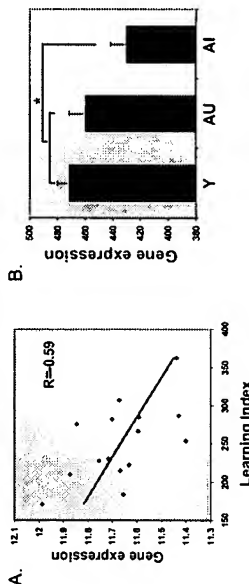
Protein extracted from whole hippocampal dissected from young rats treated with VPA (100 mg/kg/day, n = 6) or saline (n = 6) for 14 days was separated by SDS-PAGE electrophoresis and transferred to membrane using standard techniques. Blots were probed with BiP (Stressgen, 1:5000) or calreticulin (BD Biosciences, 1:2500) antibodies and visualized with HRP conjugated 2° Abs and ECL Plus western kit. Blots were then scanned using a PhosphorImager system. Data were expressed as mean ± SEM. \*p < 0.05, \*\*p < 0.01. Error bars represent SEM.

## VALPROATE IMPROVES PERFORMANCE IN COGNITIVELY-IMPAIRED AGED RATS



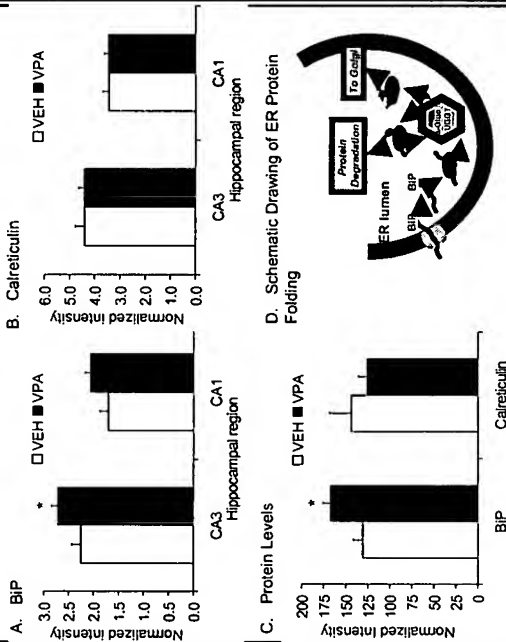
**FIGURE 1** Memory-impaired aged rats that received chronic treatment with VPA or saline performed at a comparable level at the end of the training phase of massed trials, but those treated with a high dose of VPA showed less forgetting after a 6-hr delay compared to their counterparts treated with either saline or a low dose of the drug. Rats treated with VPA at 100 mg/kg/day (VPA 100, n = 13) were significantly more accurate at locating the escape platform during retention trials as assessed by path length (A) and escape latency (B) than those treated with saline (SAL, n = 17) or VPA at 50 mg/kg/day (VPA 50, n = 9), *ps* < .05. Background performance was matched for learning index (*F* < 1), and no differences were seen between groups in cued training performance carried out at the end of the study.

## DOWNREGULATION OF ENDOPLASMIC RETICULUM CHAPERONE, BiP, IN THE CA3 OF AGED RATS WITH COGNITIVE IMPAIRMENT



**FIGURE 2** (A) Gene microarray analysis showed a negative correlation between BiP expression in the CA3 of the hippocampus and learning ability in aged rats. (B) Using a different set of animals, *in situ* hybridization analysis confirmed a significant decrease in BiP mRNA expression in aged rat with cognitive impairment compared to young and age-matched control rats with intact cognition. See Poster 95.21 / UU26 for further details.

## VALPROATE INCREASES BiP/GRP78 EXPRESSION IN THE AGED HIPPOCAMPUS



**FIGURE 3** mRNA expression levels of BiP/GRP78 (A) and calreticulin (B) in the CA3 and CA1 of the aged hippocampus after drug treatment. Cognitively impaired aged rats treated with VPA (100 mg/kg/day, n = 11) expressed significantly higher levels of BiP in the CA3 (*p* < .05) and marginally so in the CA1 than those treated with vehicle saline (n = 7). No differences in calreticulin expression between treatment conditions were detected in either region of the hippocampus. (C) Analysis of protein levels confirmed this pattern of findings. (D) Molecular chaperones in the endoplasmic reticulum (ER) such as BiP, calreticulin (Cr), and calnexin

## DISCUSSION

- Chronic treatment of sodium valproate improved spatial memory retention in cognitively impaired aged rats.
- Valproate treatment, at the dose found to improve cognition, effectively elevated the BiP level in the CA3 of the hippocampus.
- This elevation can promote correct protein folding and guard against excitotoxicity, and may be an important mechanism that confers benefit on the performance of hippocampal-dependent cognition in an animal model with features of eMCI.

## ACKNOWLEDGMENTS

Supported by NIA (P01-AG-09973) to MG.

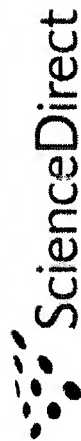


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Original article

## The effects of antiepileptic drugs on spatial learning and hippocampal protein kinase C $\gamma$ in immature rats

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Received 18 June 2001; revised 13 December 2001; accepted 21 December 2001. Available online 6 February 2002.

**Abstract**

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This study was conducted to determine if alterations in hippocampal protein kinase C (PKC)  $\gamma$  is one of the cellular mechanisms by which conventional antiepileptic drugs affect learning and memory. Wistar Rats (21-day-old) were divided into five groups: (1) control (no training and drugs); (2) training group (no drugs); (3) phenobarbital (PB) group; (4) carbamazepine (CBZ) group; and (5) valproate (VPA) group. A hippocampus dependent learning task (spatial changing learning) was used in the latter four groups lasting a total of 10 days. Correct responding rate of training group was significantly higher ( $P < 0.05$ ) than in the PB, CBZ and VPA group. The PKC  $\gamma$  staining intensity in hippocampal CA1–2 region of training group was significant greater than that of the control and PB group. There was no difference in staining intensities between the CBZ, VPA group or training group. The amount of PKC  $\gamma$  located in plasma membrane of hippocampal neurons was significantly higher in the training group ( $P < 0.05$ ) than the control, PB and VPA groups. No differences were found between the training and CBZ group. Lastly, the amount of PKC  $\gamma$  in cytosol of hippocampus did not significantly differ between any of the five groups. These results indicate that the three antiepileptic drugs used in this study all disturbed the spatial learning of immature rats. Spatial learning was concomitant with activation of PKC  $\gamma$  in hippocampal neurons. PB and VPA likely adversely affect learning and memory by interfering with PKC  $\gamma$  activation, whereas CBZ may act by a different mechanism, possibly in the post-translocation process or by a PKC  $\gamma$  independent pathway.

**Author Keywords:** Learning; Memory; Antiepileptic drug

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**Brain and Development**  
Volume 24, Issue 2, March 2002, Pages 82-87

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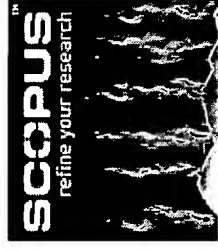
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